Pedobiologia **20**, 411–416 (1980)

Department of Microbiology, Eötvös Loránd University, Budapest

# Studies on the intestinal actinomycete flora of *Eisenia lucens* (Annelida, Oligochaeta)

Enrique Contreras

(Accepted: 15. 09. 79)

Key words: *Eisenia lucens*, *Streptomyces*, gut-flora, earthworm bacteriology

## 1. Introduction

Ruschmann (1953) and later Schütz & Felber (1956) were the first to observe the now generally known phenomenon that actinomycetes are capable of rapid growth in the digestive canal of earthworms, forming thereby an important co-dominant fraction in the gut microbiota of these animals. Further to this, as implied already by Ruschmann (1953), the large actinomycete population densities in the earthworm's digestive canal may considerably influence also the species composition of the total gut bacterial community, above all through the antibiotic activities of certain *Streptomyces* and *Nocardia* species capable of colonizing many micro-habitats of the gut milieu. At present very little is known about the ecology and biochemical capabilities of the earthworm's gut actinomycete communities, but there is reason to postulate their active participation in the transformation of organic materials, in formation of clay-humus complexes and in production of metabolic by-products ("biochemical cement") responsible for water-stability of coprogenic aggregates. Information has also been scanty on the taxonomic composition at species level of the gut actinomycete populations of the individual earthworm species although a precise knowledge of this would be indispensable for comparative investigations between the gut microbiotas of earthworms of different ecological behaviour.

Ignorance of the taxonomic-ecological aspects in the research work on the biology of earthworm's gut-actinomycetes can be excused by the considerable difficulties involved, until quite recently, in the identification of species of these organisms. Only the results of the International Streptomyces Project published by Shirling & Gottlieb (1966; 1968a, b; 1969; 1972) did open new perspectives in this respect and make the more precise identification of streptomycetes possible, which otherwise are the predominant actinomycetes in the gut of earthworms.

The present paper reports on the species composition of the gut actinomycete community of *Eisenia lucens*, an earthworm species of particular ecological behaviour, the gut-*Vibrio*-community of which was investigated and described by Márialigeti (1979).

## 2. Material and method

Sampling site: Natural forest in the mountainous region of Lillafüred (North-Eastern Hungary).

Time of sampling: Gut content samples were collected at the sampling site on two occasions, 26th September and 18th October 1977.

Sampling procedure: Specimens of *E. lucens* Waga, 1857, collected from decaying trunks of *Fagus silvatica* (beech) were washed quickly in sterile tap water, and disinfected superficially with Neomagnol (Sodium sulfamino-chloratum) solution. The fresh gut content material was obtained by massage of the animal's body with sterile forceps. The gut contents were collected into sterile Petri-dishes, and transported in portable refrigerator to the laboratory within 4 hrs.

Isolation of actinomycetes: The gut content samples were suspended and serially diluted in sterile tap water (range: $2 \times 10^{-3} - 2 \times 10^{-11}$), and plated at 0.1 ml amounts on starch-casein agar (soluble starch, Difco 10.0 g; casein, Difco, 1.0 g; $Na_2HPO_4$, 0.5 g; agar-agar, Difco purified, 15.0 g; distilled water 1000 ml; *p*H 7.2) and on glycerol-arginine agar (arginine-HCL, Difco, 10.0 g; glycerol, Difco, 12.5 g; $K_2HPO_4$, 1.0 g; NaCl, 1.0 g; $MgSO_4 \cdot 7\,H_2O$, 0.5 g; trace element solution, 1.0 ml; agar-agar, Difco purified, 15.0 g; distilled water 1000 ml; $p$H 7.0). After incubation for six days at 28 °C, the developed colonies of actinomycetes were randomly isolated and transferred on oatmeal agar slants (oatmeal, 40.0 g; agar-agar, Difco purified 20.0 g; distilled water 1000 ml; $p$H 7.2; sterilized at 121 °C), The isolates were numbered serially, incubated in thermostat at 28 °C, and stored in the refrigerator at 3 °C until use.

Purification of the strains: One loopful of each isolate was plated onto glycerol-glycine agar (glycerol, Difco, 20.0 g; glycine, 2.0 g; $K_2HPO_4$, 1.0 g; NaCl, 2.0 g; $MgSO_4 \cdot 7\,H_2O$, 0.5 g; $FeSO_4$, 0.1 g; $CaCO_3$, 0.2 g; agar-agar, Difco purified, 18.0 g; distilled water 1000 ml; $p$H 7.2; sterilized at 121 °C) in 3 Petri-dishes. After incubation for six days at 28 °C, from the distinctly growing colonies of similar morphology pure cultures were reisolated on oatmeal agar slants. These were maintained by continuous transfers, and were regularly checked for purity.

Taxonomical identification of the isolated strains: The diagnostic descriptions of the isolated strains of *Streptomyces* were carried out using the methods and criteria proposed and introduced by the ISP (Shirling & Gottlieb 1966): morphological sections of Shirling & Gottlieb; colour wheels developed by Tresner & Backus; determination of carbon utilization pattern on Pridham & Gottlieb's basal salts medium; indicator pigments detected by observing the effect of 0.05 N NaOH and 0.05 N HCl on the colour of the substrate mycelium; morphological observations on the following standard media: yeast extract — malt extract agar; oatmeal agar; inorganic salts — starch agar; glycerol-asparagine agar; tests for melanoid pigment production on tyrosine agar and peptone — yeast — iron agar; surface morphology of spores was investigated in electron-microscope at magnifications approx. 3000—7000 x, etc. For systematic identification at species level the determining key described by Szabó et al. (1975) was used. The identified strains of our collection were then directly compared with the following authentic strains of closely related species: *S. annulatus* ATCC 3307; *S. badius* INA N 1203/53; *S. candidus* ATTC 4878; *S. chrysomallus* ATCC 11523; *S. citroflurescens* FBUA 438; *S. citreus* ATCC 10974; *S. coelicolor* FBUA 491; *S. felleus* BUCSAV 6,2; *S. fimicarius* CBS Duché; *S. gougeroti* ATCC 10975; *S. griseobrunneus* ISP 5066; *S. griseoalbus* INA N 1875/54; *S. griseus* BUCSAV 7,17; *S. griseus* INA H-12; *S. griseus* Par-2; *S. griseus* IMRU 3496; *S. intermedius* ATCC 3329; *S. levoris* S-63; *S. levoris* ISP 5202; *A. levoris* G-252; *S. lipmanii* ATCC 3331; *S. microflavus* INA 3332; *S. michiganensis* A-88; *S. michiganensis* A-115; *S. odorifer* ATCC 6246; *S. praecox* ATCC 3374; *S. rubiginosohelvolus* INA 10/53; *S. rutgersensis* ATCC 3350; *S. setonii* BUCSAV 19,3; *S. willmorei* ATCC 6867.

## 3. Results

According to the plate count estimates of the relative frequency of actinomycetes in the digestive canal of *E. lucens* is considerably lower than in the gut of other species of earthworms that live in true soil habitats. We estimate their proportion in the gut flora of *E. lucens* characterized by the predominant occurrence of *Vibrio* type organisms to less than 10 per cent of the total plate count.

On a total of 145 *Streptomyces*, 15 different Micromonospora-type and 5 *Nocardia*-like strains were isolated from the gut content samples collected from *E. lucens* specimens on 26. 9. and 18. 10. 1977. A total of 122 *Streptomyces* from among the 145 isolated ones derived from the first and second sampling, proved to be the members of a single species. Our further studies were focused in the first line on this dominant organism of the gut streptomycete flora. However the identification of the species position, of the relatively rarely occurring other streptomycete gut isolates was also carried out on the basis of Szabó's determining key but without any subsequent comparison with related authentic strains.

The following species distribution was established among the isolated 145 gut streptomycete strains:

| | |
|---|---|
| 1. *S. lipmanii* (Waksman et Curtis) Waksman et Henrici, 1948 | 122 strains |
| 2. Not nearer identified *Streptomyces* spp. | 5 strains |
| 3. Related to *S. spadicis* | 3 strains |
| 4. *S. roseolus* (Preobrazhenskaja et Sveshnikova) Pridham, Hesseltine et Benedict, 1958 | 1 strain |
| 5. Related to *S. aureomonopodiales* | 1 strain |
| 6. *S. xantophaeus* | 1 strain |
| 7. *S. flaveolus* (Waksman) Waksman et Henrici, 1948 | 1 strain |
| 8. *Streptomyces* sp. (*Ochroleucus* group) | 2 strains |
| 9. *Streptomyces* sp. (*Parvus* group) | 3 strains |
| 10. *Streptomyces* sp. (*Griseus* group) | 1 strain |
| 11. *Streptomyces* sp. (*Flavochromogenes* group) | 1 strain |
| 12. Undetermined *Streptomyces* sp. | 3 strains |
| 13. *S. parvulus*-like strain | 1 strain |

It follows that only one single species was encountered, at a high frequency of occurrence, on both occasions in the gut content samples; all other *Streptomyces*, *Micromonospora* and *Nocardia*-type organisms found were only accidental members (presumedly contaminants) of the gut microflora, deriving probably from the consumed food materials. The predominant member of the gut actinomycete population was identified as *Streptomyces lipmanii* on the basis of the ISP-criteria. Although the precise taxonomic position of this species within the large "*Griseus*" group of streptomycetes to which it belongs is still not fully understood, it could be established with certainity that the gut-streptomycete population was represented by the predominance of a single species.

Remarkably, this *S. lipmanii* gut-population did not even show a notable variability in the ISP-criteria. Electron microscopic investigations revealed that all 122 strains formed spore chains of similar type (Rectus-flexibilis), coated by a smooth surface layer (membrane). No difference was found in the cultural-morphological properties and carbon source utilization spectra of the isolated 122 *S. lipmanii* strains (Table 1). The ISP-criteria were used for direct laboratory comparison of all our freshly isolated *S. lipmanii* strains with 30 authentic museum strains of *Streptomyces* belonging also to the "*Griseus*"-group (see list of the latters; chapt. 2). The result of this comparison confirmed the validity of the identification based on Szabó's key: only a single type strains, *S. lipmanii* ATCC 3331, showed a complete identity (at least on the basis of ISP criteria) with our gut isolated (Table 2).

It is remarkable, that although the diagnostic features and the C-source utilization spectra given by us for the investigated 30 authentic strains (Table 2) are generally identical with those which were published also in the ISP descriptions (Shirling & Gottlieb 1968a, b; 1969; 1972) for them, but in some cases our investigations resulted in differing

Table 1. ISP-diagnostic features of 122 *Streptomyces lipmanii* strains isolated from gut content samples of *Eisenia lucens*

| Features investigated | Media | Results |
|---|---|---|
| Sporophore morphology (Pridham's morphological sections) | Yeast extract-malt extract agar (Med. 2) | Rectiflexibiles |
| | Oatmeal agar (Med. 3) | Rectiflexibliles |
| | Inorganic salts-starch agar (Med. 4) | Rectiflexibiles |
| The mass colour of sporulating aerial mycelium (colour series of Tresner & Backus) | Med. 2 | Yellow (2ba) |
| | Med. 3 | Yellow (1cb) |
| | Med. 4 | Yellow (2db) |
| Pigmentation of the substrate mycelium (ISP color series) | Med. 2 | Yellow-brown: no distinctive pigments |
| | Med. 3 | Yellow-brown |
| | Med. 4 | Yellow-brown |
| Soluble pigments | Med. 2, 3 and 4 | None |
| Response of substrate mycelium pigments ot *p*H change | Med. 2, 3 and 4 | None |
| Melanoid pigment production | Peptone-yeast-iron agar | None |
| | Tyrozine agar | None |
| Spore surface morphology at a magnification of about 8000 × | Med. 3 | Smooth |
| D-glucose | Pridham & Gottlieb's basal salts medium | Positive |
| L-arabinose | | Negative |
| Sucrose | | Negative |
| D-xylose | | Positive |
| I-inositol | | Negative |
| D-mannitol | | Positive |
| D-fructose | | Positive |
| Rhamnose | | Positive |
| Raffinose | | Negative |

Table 2. A comparison of the ISP diagnostic features of 30 authentic, mostly tape strains of *Streptomyces* spp. *(Griseus*-series*)* with the summarized data of 122 *S. lipmanii* strains isolated from the digestive canal of *Eisenia lucens**)

| Specific epithet and designation of strains | Sporophore morphology | No of spores per chain | Spore surface | Spore mass colour | Colour-series of vegetative mycelium | Soluble pigment | Melanoid pigment | | Change of substrate mycelium pigment | | C-source utilization spectra | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | M6 | M7 | HCl | NaOH | D-glucose | L-arabinose | Sucrose | D-xylose | i-inositol | D-mannitol | D-fructose | Rhamnose | Raffinose |
| Summarized data of our 122 gut-content isolates | RF | 10–50 | Sm | Y | Y-b | – | – | – | – | – | ++ | – | – | ++ | – | ++ | ++ | ++ | – |
| *S. annulatus* ATCC 330 | RF | 3–10 | Sm | Y, W | Y-b | – | – | – | ? | – | ++ | ++ | – | ++ | + | ++ | ++ | – | – |
| *S. badius* INA N 1203/53 | RF | 10–50 | Sm | Y | Y-b | – | – | – | – | – | ++ | ++ | – | ++ | – | ++ | ++ | – | ++ |
| *S. candidus* ATCC 4878 | RF | 3–10 | Sm | Y | Y-b | – | – | – | ? | ? | ++ | ++ | – | ++ | + | ++ | ++ | – | ++ |
| *S. chrysomalus* ATCC 11523 | RF | 10 | Sm | Y | Y-b | – | – | – | – | – | ++ | ++ | – | ++ | – | ++ | ++ | – | ++ |
| *S. citreofluorescens* FBUA 438 | RF | 10–50 | Sm | Y | Y-b | – | – | – | – | – | ++ | ++ | – | ++ | – | ++ | ++ | ++ | – |
| *S. citreus* ATCC 10974 | RF | 10–50 | Sm | Y | Y-b | – | – | – | – | – | ++ | ++ | ++ | ++ | ? | ++ | ++ | – | ? |
| *S. coelicolor* FBUA 491 | RF | 10–50 | Sm | G | Y-b + g | viol. | – | – | + | + | ++ | ++ | – | ++ | ++ | ++ | ++ | ++ | ++ |
| *A. felleus* BUCSAV 6,2 | RF | 10–50 | Sm | Y, W | Y-b | – | – | – | – | – | ++ | ? | – | ++ | – | ++ | ++ | ++ | – |
| *S. fimicarius* CBS Duche | RF | 10–50 | Sm | W? | Y-b | – | – | – | + | – | ++ | ++ | – | ++ | – | ++ | ++ | ++ | – |
| *S. gougeroti* ATCC 10975 | ? | ? | ? | ? | Y-b | – | – | – | – | – | ++ | ++ | – | + | ++ | ++ | ++ | – | – |
| *S. griseobrunneus* ISP 5066 | RF | 10–50 | Sm | Y | Y-b | – | + | – | – | – | ++ | – | – | ++ | – | ++ | ++ | – | – |
| *S. griseoloalbus* INA N 1875/54 | RF | 10–50 | Sm | Y | Y-b | – | – | – | – | – | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | – |

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. griseus*<br>BUCSAV 7,17 | RF | 10–50 | Sm | Y | Y-b | — | — | ? | — | — | ++ | — | — | ++ | — | ++ | ++ | — | — |
| *S. griseus*<br>INA II-12 | RF | 10–50 | Sm | Y | Y-b | — | — | ? | — | — | ++ | ++ | — | — | — | ++ | ++ | ++ | — |
| *S. griseus*<br>Par.-2 | RF | 10–50 | Sm | Y | Y-b | — | — | ? | — | — | ++ | — | — | ++ | — | ++ | ++ | — | — |
| *S. griseus*<br>IMRU 3496 | RF | 10–50 | Sm | Y | Y-b | — | — | ? | — | — | ++ | — | ± | ++ | — | ++ | ++ | — | — |
| *A. levoris*<br>ATCC 3329 | RF | 3–10 | Sm | Y | Y-b | — | — | — | — | — | ++ | ++ | ++ | ++ | — | ++ | ++ | — | — |
| *S. intermedius*<br>S-63. Biogal | RF | 10–50 | Sm | Y | Y-b | — | — | — | — | — | ++ | ++ | — | + | — | ++ | ++ | — | — |
| *S. levoris*<br>ISP 5202 | RF | 10–50 | Sm | Y | Y-b | — | — | — | — | — | ++ | ++ | ± | ++ | — | ++ | ++ | — | — |
| *A. levoris*<br>G-252 ESM | RF | 10–50 | Sm | Y, W | Y-b | — | — | — | — | — | ++ | ++ | — | ++ | — | ++ | ++ | — | — |
| *S. lipmanii*<br>ATCC 3331 | RF | 10–50 | Sm | Y | Y-b | — | — | — | — | — | ++ | — | — | ++ | — | ++ | ++ | ++ | — |
| *A. microflavus*<br>INA 3332 | RF | 10–50 | Sm | Y, G | Y-b | — | — | — | — | — | ++ | — | — | ++ | — | ++ | ++ | — | — |
| *S. michiganensis*<br>A 88 | RF | 10–50 | Sm | Y | Y-b | — | + | — | — | — | ++ | — | — | ++ | + | ++ | ++ | — | ± |
| *S. michiganensis*<br>A-115 | RF | 50 | Sm | Y | Y-b | yell. | + | — | — | — | ++ | — | — | ++ | ? | ++ | ++ | — | ± |
| *S.* odorifer<br>ATCC 6246 | RF | 10–50 | Sm | Y | Y-b | — | — | — | — | — | ++ | ++ | ++ | ++ | — | ++ | + | — | — |
| *S. praecox*<br>ATCC 3374 | RF | 10–50 | Sm | W | Y-b | — | — | — | — | — | ++ | ++ | — | ++ | — | ++ | ++ | ++ | — |
| *A. rubiginosohelvolus*<br>INAN 10/53 | RF | 10–50 | Sm | Y | Y-b | — | — | — | — | — | ++ | ++ | — | ++ | — | ++ | ++ | ++ | — |
| *S. rutgersensis*<br>ATCC 3350 | RF? | 10–20 | Sm | Y | Y-b | — | ? | — | — | — | ++ | — | — | ++ | — | ++ | ++ | — | — |
| *A. setonii*<br>BUCSAV 19,3 | RF | 3–10 | Sm | Y | Y-b | — | — | — | — | — | ++ | ++ | — | ++ | — | ++ | ++ | ++ | — |
| *S. willmorei*<br>ATCC 6867 | RF | 10–50 | Sm | Y | Y-b | — | — | — | — | — | ++ | ± | ± | ++ | — | ++ | ++ | ++ | — |

Note. — Authentic strains were investigated synchronously and compared directly with our gut-content isolates.
*) Abb.: Y = Yellow-series; W = Whiteseries; Y-b = Yellow-brown series; Y-b+g = Y-b+ green; RF = Rectiflexibiles; Sm = Smooth.

data. E.g. in raffinose and rhamnose utilization of *S. candidus* ATCC 4878, *S. chrysomallus* ATCC 11523, *S. coelicolor* FBUA 491 etc. The reason of these differences lie perhaps in temporal variation of these strains.

## 4. Discussion

The present studies have undoubtedly shown that more than 70 % of the gut actinomycete flora of *Eisenia lucens* were represented by the members of a single species: *S. lipmanii* (Waksman et Curtis) Waksman et Henrici, which otherwise is a relatively unfrequent organism in nature. This accords well with the earlier observations of other workers (Szabó 1974) that usually only few species, or occasionally only a single one of the gut actinomycete population can occur in the intestinal milieu forming relatively large population densities and occupy there a prominent community position.

Further studies are, of course, required to clarify the nature of the cooperative interrelationships of *S. lipmanni* with other frequent members of the intestinal bacterial flora (e.g. with vibrios) and with the earthworm host itself. It is merely a taxonomic problem whether or not the type or authentic strains of *S. griseus* INA H-12, *S. willmorei* ATCC 6867, *S. rubiginosohelvolus* INA N 10/53 and *S. lipmanii* ATCC 3331, which are very similar to one another, represent only individual members of a single species *(S. lipmanii)*. At all events it seems very questionable that the earlier concept of some workers, according to which actinomycetes of the consumed food (soil) materials grow, multiply, and become co-dominant in the earthworm's gut without any particular selection, has a general validity. At least in the case of *Eisenia lucens* not at all.

## 5. Summary

In the intestinal canal of *Eisenia lucens* actinomycetes form a less large fraction of the gut population composed pre-dominantly of *Vibrio* type bacteria. The results of the taxonomic analysis of two representative samples (a total of 165 strains) of the gut actinomycete community show that streptomycetes form near 90 percent of this community, within which one single species — *Streptomyces lipmanii* — is the absolute pre-dominant organisms. This statement has let us to conclude that the gut milieu has a particular selective influence. The identification of 122 freshly isolated strains of *S. lipmanii* was carried out on the basis of their direct comparison with 30 authentic, mostly type strains of closely related species using the methods and criteria of the International Streptomyces Project.

## 6. References

Márialigeti, K., 1979. On the community-structure of the gut-microbiota of *Eisenia lucens* (Annelida, Oligochaeta). Pedobiologia **19**, 213—220.

Ruschmann, G., 1953. Über Antibiosen und Symbiosen von Bodenmikroorganismen und ihre Bedeutung für die Bodenfruchtbarkeit. Z. Acker- und Pflanzenbau **96**, 201—218.

Schütz, W., & E. Felber, 1956. Welche Mikroorganismen spielen im Regenwurmdarm bei der Bildung von Bodenkrümeln eine Rolle? Z. Acker- und Pflanzenbau **101**, 471—476.

Shirling, E. B., & D. Gottlieb, 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. **16**, 313—340.

— — 1968a. Cooperative description of type cultures of *Streptomyces*. II. Species description from first study. Int. J. Syst. Bacteriol. **18**, 69—189.

— — 1968b. Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. **18**, 279—392.

— — 1969. Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Int. J. Syst. Bacteriol. **19**, 391—512.

— — 1972. Cooperative description of type strains of *Streptomyces* (V. Additional descriptions) Int. J. Syst. Bacteriol. **22**, 265—394.

Szabó, I. M., M. Marton, I. Buti & C. Fernandez, 1975. A diagnostic key for the identification of "species" of *Streptomyces* and *Streptoverticillium* included in the International Streptomyces Project. Act. Bot. Acad. Sci. Hung. **21**, 387—418.

— 1974. Microbial communities in a forest-rendzina ecosystem. Akad. Kiadó, Budapest.

Tresner, H. D., & E. J. Backus, 1963. System of color wheels for *Streptomycetes* taxonomy. Appl. Microbiol. **11**, 335—338.

Address of the author: Enrique O. Contreras, Mikrobiológiai Tanszék [Department of Microbiology], Eötvös Loránd Tudományegetem [Eötvös Loránd University], Múzeum krt. 4a., H - 1088 Budapest [Hungary].